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RESEARCH PAPER

Calcium and calcium-dependent protein kinases are involved in nitric oxide- and auxin-induced adventitious root formation in cucumber

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Abstract

A few years ago it was demonstrated that nitric oxide (NO) and cGMP are involved in the auxin response during adventitious root (AR) formation in cucumber (*Cucumis sativus*). More recently, a mitogen-activated protein kinase cascade was shown to be induced by IAA in a NO-dependent, but cGMP-independent, pathway. In the present study, the involvement of Ca^{2+} and the regulation of Ca^{2+}-dependent protein kinase (CDPK) activity during IAA- and NO-induced AR formation was evaluated in cucumber explants. The effectiveness of several broad-spectrum Ca^{2+} channel inhibitors and Ca^{2+} chelators in affecting AR formation induced by IAA or NO was also examined. Results indicate that the explants response to IAA and NO depends on the availability of both intracellular and extracellular Ca^{2+} pools. Protein extracts from cucumber hypocotyls were assayed for CDPK activity by using histone IIIS or syntide 2 as substrates for in-gel or *in vitro* assays, respectively. The activity of a 50 kDa CDPK was detected after 1 d of either NO or IAA treatments and it extended up to the third day of treatment. This CDPK activity was affected in both extracts from NO- and IAA-treated explants in the presence of the specific NO-scavenger cPTIO, suggesting that NO is required for its maximal and sustained activity. The in-gel and the *in vitro* CDPK activity, as well as the NO- or IAA-induced AR formation, were inhibited by calmodulin antagonists. Furthermore, the induction of CDPK activity by NO and IAA was shown to be reliant on the activity of the enzyme guanylate cyclase.

Key words: Adventitious roots, auxin, Ca^{2+}, Ca^{2+}-dependent protein kinase, cGMP, *Cucumis sativus*, guanylate cyclase, nitric oxide.

Introduction

The auxin indole acetic acid (IAA) regulates many aspects of plant growth and development from seed germination to fruit ripening, mediating cell division, expansion, and differentiation (Davies, 1995). However, despite the critical role of auxins throughout the plant life cycle, the molecular mechanisms underlying their action are still poorly understood. Adventitious root (AR) formation involves the development of a meristematic tissue after removal of the primary root system. The auxin IAA promotes AR formation by inducing the dedifferentiation of cells to develop new meristems. This process is essential for the propagation of woody species and for adaptation to particular environmental conditions (Moncousin, 1992). It was recently demonstrated that nitric oxide (NO) is involved in the auxin response during AR formation in cucumber (*Cucumis sativus*; Pagnussat *et al*., 2002) and that convergent and complex cGMP-dependent and -independent signalling pathways are orchestrating the formation of a new root system when the primary root is removed (Pagnussat *et al*., 2003, 2004).

Although first described as a signal molecule in animals, new and exiting roles for NO as a regulator of plant growth and developmental processes have been reported during the past few years (Beligni and Lamattina, 2001; Lamattina *et al*., 2003; Neill *et al*., 2003; Wendehenne *et al*., 2004).

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Abbreviations: AR, adventitious root; CaM, calmodulin; $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration; CDPK, Ca^{2+}-dependent protein kinase; IAA, indole acetic acid; GC, guanylate cyclase; NO, nitric oxide; PLC, phospholipase C; PM, plasma membrane.

A connection among NO, cGMP, Ca^{2+}, and calmodulin (CaM) pathways was suggested in previous studies that showed the participation of NO in light-mediated processes in plants (Beligni and Lamattina, 2000). In tobacco (*Nicotiana tabacum*) cells, cADPR was able to mimic the NO-mediated induction of defence gene expression. This cADPR effect was inhibited by ruthenium red, an inhibitor of Ca^{2+} release via cADPR/ryanodine-sensitive Ca^{2+} channels (Durner *et al*., 1998). Furthermore, 8-Br-cADPR, an antagonist of cADPR, blocked gene expression mediated by recombinant neuronal nitric oxide synthase in tobacco (Klessig *et al*., 2000). More recently, it was demonstrated that NO triggered an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) by promoting Ca^{2+} release from intracellular stores through the activation of cADPR/ryanodine-sensitive Ca^{2+} channels in fava bean (*Vicia faba*) and tobacco cells (García-Mata *et al*., 2003; Lamotte *et al*., 2004).

The second messenger inositol 1,4,5-trisphosphate (IP_3) diffuses within the cell where it encounters specific receptors allowing the Ca^{2+} stored in organelles to enter the cytosol (Bootman *et al*., 2001). There is evidence that a rise in IP_3 concentration follows auxin signalling and activates Ca^{2+} channels located in intracellular compartments such as the vacuole (Ettlinger and Lehle, 1988; Zbell and Walter-Back, 1988). Moreover, it was reported that both the auxin-induced cytosolic acidification and elongation growth were blocked by inhibiting the Ca^{2+} influx into the cytosol (Poovaiah and Reddy, 1987; Cho and Hong, 1996; Shishova and Lindberg, 1999), which indicate that some cell responses to auxins depend on activity of Ca^{2+} channels.

Available evidence supports the idea that both cADPR/ryanodine-sensitive Ca^{2+} channels and IP_3-regulated Ca^{2+} channels are functionally active in plant cells (Muir and Sanders, 1996, 1997). However, their biochemical and pharmacological properties cannot be extrapolated from experiments done with animal Ca^{2+} channel inhibitors since there is little evidence for direct effects and specificity on plant Ca^{2+} channels. Thus, an understanding of the participation and occurrence of these signalling pathways is not yet complete, since no orthologues of both types of Ca^{2+} channels were found in plants at the molecular level (Nagata *et al*., 2004).

In plants, the elevations in $[Ca^{2+}]_{cyt}$ induced by a particular stimulus are perceived and transduced by specific effectors involving Ca^{2+}-binding proteins and protein kinases that initiate downstream events leading to changes in gene expression, metabolism, and cell division and elongation (Sanders *et al*., 2002; White and Broadley, 2003; Reddy and Reddy, 2004). These proteins are also referred to as Ca^{2+} sensors and include (i) CaM, (ii) Ca^{2+}-dependent protein kinases (CDPKs), (iii) Ca^{2+}-regulated phosphatases, and (iv) annexins and integrins. CDPKs (EC 2.7.1.37) are a family of serine/threonine protein kinases that are unique to plants and protists (for reviews see Roberts and Harmon, 1992; Roberts, 1993; Harmon *et al*., 2000, 2001; Cheng *et al*., 2002; Harper *et al*., 2004; Ludwig *et al*., 2004). These enzymes contain four domains: (i) an amino terminal domain of variable length and sequence, (ii) a protein kinase catalytic domain, (iii) an autoinhibitory junction domain, and (iv) a CaM-like Ca^{2+}-binding domain in their carboxyl terminal (Harmon *et al*., 2001). CDPKs are activated by the binding of Ca^{2+} to their CaM-like domain. In resting conditions, $[Ca^{2+}]_{cyt}$ is maintained at low levels assuring a basal CDPK activity. Different biotic and abiotic stimuli trigger increases in $[Ca^{2+}]_{cyt}$, resulting in the activation of certain CDPKs and in the subsequent initiation of signalling events (Webb *et al*., 1996; Trewavas and Malhó, 1998; Hrabak, 2000). In many plant species, CDPKs are encoded by a multigene family. In cucumber, there have been isolated and characterized four partial cDNAs (Ullanat and Jayabaskaran, 2002) and one full-length cDNA (Kumar *et al*., 2004) encoding putative CDPK isoforms. Activity of CDPKs was found in various organs of cucumber seedlings and the proteins responsible for that activity were immunolocalized in the phloem sieve elements. A role for CDPKs in the Ca^{2+}-regulated phloem transport of assimilates from leaves to sink organs was suggested (Kumar and Jayabaskaran, 2004).

In the present study, the involvement of Ca^{2+} and the regulation of CDPK activity was evaluated during the IAA- and NO-induced AR formation in cucumber explants. Evidence is provided that Ca^{2+} and CDPK activity are downstream messengers in the signalling pathway triggered by auxins and NO to promote AR formation.

Materials and methods

Plant material and experimental design

Cucumber seeds (*Cucumis sativus* cv. Poinsett 76) were germinated in Petri dishes on filter papers soaked in distilled H_2O and maintained at 25 °C for 7 d with a 14 h photoperiod (photosynthetically active radiation=200 μmol m^{-2} s^{-1}). Primary roots of 7-d-old seedlings were removed and cucumber explants were maintained under the same conditions of temperature and photoperiod for up to 5 d in the presence of H_2O (control) or 10 μM of the auxin IAA (Fluka Buch, Switzerland) or 10 μM of the NO-donor sodium nitroprusside (SNP; Merck, Darmstadt, Germany). As a control, 200 μM of the specific NO-scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (cPTIO; Molecular Probes Eugene, Oregon, USA) was added together with SNP or IAA.

Treatments with Ca^{2+} chelators, Ca^{2+} channel inhibitors, and calmodulin antagonists

All the chemicals except for 1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM; Calbiochem, San Diego, USA) were purchased from Sigma-Aldrich (St Louis, USA). Before the removal of the primary root, cucumber seedlings were pretreated in Petri dishes for 2 h in the presence of 100 μM ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or 100 μM BAPTA/AM or 500 μM lanthanum chloride

($LaCl_3$) or 150 μM methoxyverapamil hydrochloride (MV) or 5 mM lithium chloride (LiCl) or 50 μM neomycin sulphate (NEO) or 50 μM ruthenium red (RR) or 100 μM nicotinamide (NA). Primary roots were then removed and whole explants were treated for 5 d in Petri dishes containing filter papers imbibed in solutions with the above compounds in the presence or absence of 10 μM IAA, or 10 μM SNP. As a control, cucumber explants were maintained in H_2O, IAA or SNP. The same experimental procedure was used to evaluate the effect of the membrane-permeable CaM antagonists chlorpromazine hydrochloride (CPZ), trifluoperazine dihydrochloride (TFP), and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) on the AR formation. To establish the inhibitor assay conditions, dose–response analysis was performed with different pretreatment times. All the inhibitors except for LiCl were assayed between 10 μM and 1 mM. In the case of LiCl, the dose–response ranged from 1 mM to 10 mM. The time of pretreatment was assayed from 2 h to 24 h. The concentrations of the inhibitors and the time of pretreatments were chosen as the minor ones that were effective. Until the 5th day no particular phenotype concerning explants growth or development could be observed and ascribed to the effect of the inhibitors. By contrast, all the explants treated with the inhibitors tested in this study looked healthy.

Protein extracts and protein determination

All the extraction procedures were performed on ice. Hypocotyls from cucumber explants were ground in a mortar with liquid nitrogen and extracted with 3 vols (3 ml g^{-1} FW) of extraction buffer (100 mM TRIS pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM PMSF, 5 μg ml^{-1} aprotinin, and 5 μg ml^{-1} leupeptin). After centrifugation at 20 000 *g*, 4 °C for 15 min, supernatants were transferred into clean tubes and immediately used for analysis. The quantification of proteins was performed according to Bradford (1976) using bovine serum albumin as standard.

In-gel protein kinase activity assay

In-gel protein kinase activity assays were performed as described previously with some minor modifications (Zhang *et al*., 2000). Extracts containing 10 μg of protein were electrophoresed on 12% SDS-polyacrylamide gels imbibed in 0.25 mg ml^{-1} of histone IIIS (Sigma-Aldrich, St Louis, USA) in the separating gel as substrate for the protein kinases. After electrophoresis, SDS was removed by washing the gel with washing buffer (25 mM TRIS, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg ml^{-1} BSA, and 0.1% Triton X-100 [v/v]) three times for 30 min each at room temperature. The protein kinases were allowed to renature in 25 mM TRIS, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4, and 5 mM NaF at 4 °C overnight with three changes of buffer. The gel was then incubated at room temperature in 30 ml of reaction buffer (25 mM TRIS, pH 7.5, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, and 2 mM $CaCl_2$ or 2 mM EGTA) with 200 nM cold ATP plus 50 μCi γ-^{32}P-ATP (6000 Ci $mmol^{-1}$) for 60 min. The reaction was stopped by transferring the gel into 5% TCA (w/v)/1% NaPPi (w/v). The unincorporated γ-^{32}P ATP was removed by washing five times with the same solution for at least 6 h. The gel was dried at 80 °C for 60 min and exposed to Kodak X-OMAT UV film. Prestained size markers (Bio-Rad, Hercules, California, USA) were used to estimate the size of the protein kinases.

In vitro *protein kinase activity assay*

The protein kinase activity was determined *in vitro* by measuring phosphate incorporation into syntide 2 (Sigma-Aldrich, St Louis, USA) following Roskoski's procedure (Roskoski, 1983) with some minor modifications. The reaction was carried out in a final volume of 30 μl. Fifteen μl of sample was assayed in a reaction mixture containing 25 μM syntide 2, 20 mM TRIS pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 5 μM $CaCl_2$ or 500 μM EGTA. The reactions were started by the addition of 100 μM cold ATP and 0.7 μCi γ-^{32}P-ATP (6000 Ci $mmol^{-1}$). After incubating at room temperature for 15 min, 25 μl of the mixture were spotted onto P81 phosphocellulose squares (Whatman, Springfield Mill, Maidstone, UK) and washed extensively by shaking in 75 mM H_3PO_4. The paper squares were dried and counted with a Liquid Scintillation Counter Beckman LS 7000 as described by Ulloa *et al*. (1991). The protein kinase activity was measured in the absence (EGTA) or presence ($CaCl_2$) of Ca^{2+}. The Ca^{2+}-dependent protein kinase (CDPK) activity was expressed as pmol ^{32}P min^{-1} mg^{-1} protein after subtracting the Ca^{2+}-independent protein kinase activity. Where indicated, 100 μM of the CaM antagonists TFP and W-7, or 50 μM of the guanylate cyclase inhibitor 6-anilino-5, 8-quinilinedione (LY83583; Sigma-Aldrich, St Louis, USA) were administered to explants. To determine the effect of the CaM antagonists, a combination of both TFP and W-7 was added to the reaction mixture and the CDPK activity was measured as described above.

Results

Ca^{2+} channel inhibitors and Ca^{2+} chelators prevent the IAA- and nitric oxide-induced adventitious root formation

Available evidence shows that generation of cGMP, cADPR, and elevations in $[Ca^{2+}]_{cyt}$ are involved in plant responses to NO (Wendehenne *et al*., 2001; García-Mata *et al*., 2003; Neill *et al*., 2003). It was previously reported that in cucumber explants, NO operates downstream of IAA promoting AR formation through the guanylate cyclase (GC)-catalysed synthesis of cGMP (Pagnussat *et al*., 2003). Taking advantage of the knowledge on Ca^{2+} participation in some NO-regulated signalling pathways, the requirement of Ca^{2+} for the IAA- and NO-induced AR formation was examined. The effectiveness was tested of several broad-spectrum Ca^{2+} channel inhibitors and Ca^{2+} chelators in reducing AR formation. The inhibitors of Ca^{2+} release from intracellular stores via cADPR/ryanodine-sensitive channels, methoxyverapamil hydrochloride (MV; Moyen *et al*., 1998; Shishova and Lindberg, 2004) and ruthenium red (RR; Price *et al*., 1994; Trewavas and Knight, 1994; Allen *et al*., 1995), were assayed. As expected, the treatment with 10 μM of the NO-donor sodium nitroprusside (SNP) or with 10 μM of the auxin IAA promoted AR formation in cucumber explants (Fig. 1). As shown in Fig. 1, the application of the inhibitors prevented the induction of AR formation by either NO or IAA (*t* test, $P < 0.05$). Since externally provided RR and verapamil has also been shown to inhibit plasma membrane (PM) Ca^{2+} channels (White, 2000; White *et al*., 2002), the effect of nicotinamide (NA), which as a product of the activity of the enzyme ADPR cyclase inhibits cADPR synthesis (Wu *et al*., 1997) was tested. Treatment of cucumber explants with NA resulted in a significant reduction in both IAA- and NO-induced AR formation (Fig. 1; *t* test, $P < 0.05$).

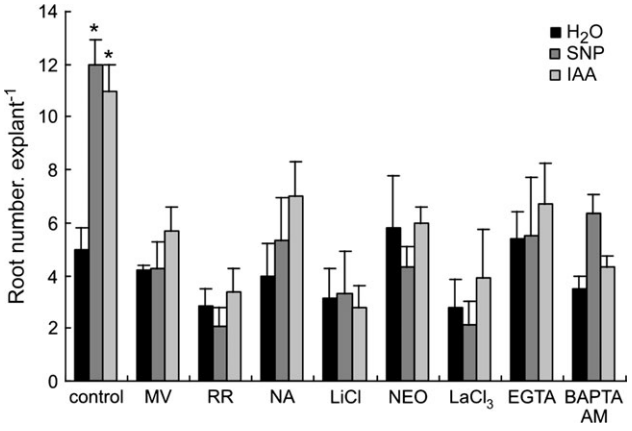

Fig. 1. Ca^{2+} is required for adventitious root formation induced by indole acetic acid (IAA) and nitric oxide (NO). Cucumber explants were incubated for 5 d with different Ca^{2+} chelators or Ca^{2+} channel inhibitors, in the presence or absence of 10 μM of the NO-donor SNP or 10 μM of the auxin IAA. Root number values are expressed as mean ±SE (n=10 explants from at least three independent experiments). Bars with asterisk are significantly different (t test, P <0.05) from their control without SNP or IAA. See Materials and methods section for the full description of the experimental procedure. MV, methoxyverapamil hydrochloride (150 μM); RR, ruthenium red (50 μM); NA, nicotinamide (100 μM); LiCl, lithium chloride (5 mM); NEO, neomycin sulphate (50 μM); $LaCl_3$, lanthanum chloride (500 μM); EGTA, ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (100 μM); BAPTA/AM, 1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (100 μM).

Ca^{2+} efflux from intracellular stores can also be mediated by the well-described ligand IP_3 (Allen *et al*., 1995). Therefore, the blockers of IP_3-regulated Ca^{2+} channels lithium chloride (LiCl, which suppress IP_3 cycling and signalling by inhibiting inositol-1-phosphatase; Gillaspy *et al*., 1995; Liang *et al*., 1996) and neomycin sulphate [NEO, which inhibits IP_3 formation by avoiding the binding of the enzyme phospholipase C (PLC) to its substrate phosphatidylinositol 4,5-bisphosphate; Munnik *et al*., 1998] were administered to cucumber explants in the presence or absence of NO or IAA. Again, these inhibitors significantly suppressed the AR formation induced by IAA or NO (Fig. 1, t test, P <0.05).

Since Ca^{2+}-mediated signalling pathways are thought to depend on the activation of PM Ca^{2+} channels and the consequent influx of extracellular Ca^{2+} into cells for their function (Sanders *et al*., 2002), the effect of the Ca^{2+} channel inhibitor lanthanum chloride ($LaCl_3$; Tlalka and Gabrys, 1993; Huang *et al*., 1994; Bush, 1995; van der Meulen *et al*., 1996; Clayton *et al*., 1999) on the induction of AR formation was also analysed. The results presented in Fig. 1 indicate that $LaCl_3$ was able to prevent the stimulating effect of both NO and IAA on AR formation (t test, P <0.05). However, these results should be interpreted with some caution. Even though lanthanum is used as a blocker of PM Ca^{2+} channels, it can also block intracellular Ca^{2+} channels. It has been observed that lanthanum enters the cells when provided in milimolar concentrations for periods greater than 1 min (Peeters *et al*., 1989; Quiquampoix *et al*., 1990; Zha and Morrison, 1995). Nevertheless, the involvement of extracellular and intracellular Ca^{2+} was further confirmed by the use of the membrane-impermeable Ca^{2+} chelator ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and the membrane-permeable Ca^{2+} chelator 1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM; van der Luit *et al*., 1999; Cousson, 2003), respectively. Figure 1 shows that these compounds significantly blocked both the auxin- and NO-induced AR formation (t test, P <0.05).

CDPK activity is induced by IAA in a NO-mediated pathway

In order to study a possible link between Ca^{2+} and downstream signalling sensors, the Ca^{2+}-dependent protein kinase (CDPK) activity in cucumber explants was measured. To characterize the CDPK activity present in soluble extracts of cucumber hypocotyls, in-gel protein kinase assays were performed in the presence of an exogenous substrate histone IIIS (Roberts and Harmon, 1992). Only one activity band that was Ca^{2+}-dependent was detected in the in-gel analysis. This band corresponded to a protein kinase with an apparent molecular mass of approximately 50 kDa (Fig. 2A, B). Since no signal was detected in the absence of Ca^{2+} (SNP+EGTA and IAA+EGTA; Fig. 2A, B, respectively), the 50 kDa phosphorylating signal was attributed to a CDPK activity. This activity was detected after the first day of both SNP and IAA treatments and it extended up to the third and the fourth days of treatment, respectively (Fig. 2A, B). When analysed, the activation of this 50 kDa CDPK was delayed in explants treated with SNP plus the specific NO-scavenger cPTIO (Fig. 2A). Furthermore, this activity band was faint in both the third and the fourth day of IAA+cPTIO treatment, while it was clearly visible in the case of the IAA treatment (Fig. 2B). Under these experimental conditions, no activity could be detected in both control H_2O- and cPTIO-treated explants (Fig. 2C).

A previous study showed that within 3 d after removal of the primary root system, adventitious root primordia formation was detected in explants treated with IAA or NO, while at that time cell proliferation was barely detected and no root primordia could be observed in H_2O-treated explants (Pagnussat *et al*., 2004). Figure 2D displays the kinetics of AR formation in cucumber explants treated with H_2O, NO or IAA in the presence or absence of cPTIO. After 4 d of treatment, the number of adventitious roots was 400% higher in NO- and IAA-treated explants than in the control ones. As was already demonstrated, the scavenging of NO with cPTIO results in a decrease in adventitious root number (Fig. 2D; Pagnussat *et al*., 2002).

To further confirm that the 50 kDa CDPK activity detected during the IAA- and NO-induced AR formation

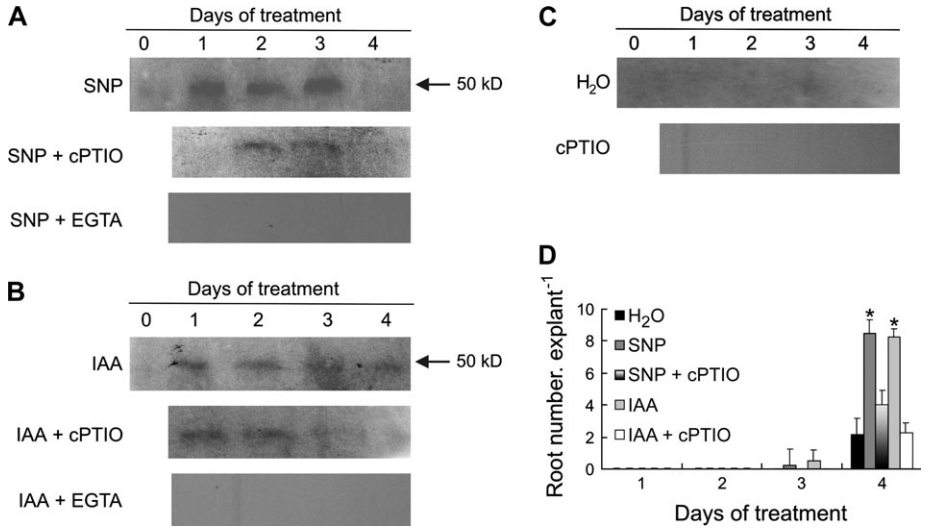

Fig. 2. In-gel CDPK activity is induced by IAA and NO during adventitious root formation. (A) Cucumber explants were incubated for different times with 10 μM of the NO-donor SNP or with SNP plus 200 μM of the specific NO-scavenger cPTIO. In-gel protein kinase activity assays were assayed in total soluble extracts from the hypocotyls of those explants in the presence (SNP) or absence of Ca^{2+} (SNP+EGTA). The arrow indicates an apparent molecular mass of approximately 50 kDa. (B) Cucumber explants were incubated for different times either with 10 μM IAA or with IAA plus 200 μM cPTIO. Protein kinase activity was measured in the presence (IAA) or absence of Ca^{2+} (IAA+EGTA). The arrow indicates an apparent molecular mass of approximately 50 kDa. (C) Cucumber explants were incubated for different times with either H_2O or 200 μM of cPTIO and protein extracts from the hypocotyls of those explants were used for in-gel protein kinase activity assays. (D) Cucumber explants were treated with 10 μM SNP or 10 μM IAA in the presence or absence of 200 μM cPTIO. Root number was measured during 4 d of treatment every 24 h and the values are expressed as mean ±SE (n=10 explants from at least three independent experiments). Bars with asterisk are significantly different (t test, P <0.05) from the H_2O-treatment. Protein kinase activity was measured using histone IIIS as substrate copolymerised in the gels.

was univocally a member of the cucumber CDPK family, in-gel protein kinase experiments were performed in extracts from explants treated with SNP or IAA in the presence of a combination of both CaM antagonists trifluoperazine dihydrochloride (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7). TFP was reported to inhibit plant CDPKs by competing with the binding of Ca^{2+} to their CaM-like domain (Harmon *et al*., 1987; Roberts and Harmon, 1992; Li *et al*., 1998; Chico *et al*., 2002). W-7 is widely used to study the role of CaM in different signalling pathways and it was also shown to be a rather non-specific inhibitor of Ca^{2+}-dependent enzymes (Lam *et al*., 1989; Anil and Rao, 2000). No phosphorylating signal could be detected when in-gel protein kinase activity assays were carried out with extracts from inhibitor-treated explants (data not shown), indicating that the 50 kDa CDPK activity might posses CaM-like Ca^{2+}-binding domains.

The results obtained from in-gel protein kinase activity assays might be conditioned by the ability of each CDPK to resist denaturalization and subsequent renaturalization. Thereby, *in vitro* protein kinase activity assays were used to confirm and quantify the effect of the CaM antagonists TFP and W-7. Table 1 shows that, in the protein extracts from explants treated with SNP plus a combination of TFP and W-7 (SNP+INH), the CDPK activity was totally inhibited at the first day and more than 40% between the second and the fourth days. Furthermore, the *in vitro* CDPK activity was strongly inhibited in extracts from IAA+INH-treated cucumber explants (Table 1).

CDPK activity is required for adventitious root formation

It was also studied how the inhibition of the CDPK activity affects both the IAA- and NO-induced AR formation. Cucumber explants were incubated with H_2O, IAA, or the NO-donor SNP either in the presence or absence of 100 μM of the CaM antagonists TFP or W-7. The CaM antagonist chlorpromazine hydrochloride (CPZ) was also assayed. As shown in Fig. 3A and B, all the CaM antagonists assayed were able to prevent the promotion of ARs by IAA and NO (t test, P <0.05). The inhibitory effect displayed by TFP was stronger than those of W-7 and CPZ (Fig. 3A). The potency of these drugs is in accordance with previously published reports in which the efficacy of TFP, W-7, and CPZ in inhibiting the activity of CDPKs was evaluated (Abo-El-Saad and Wu, 1995; MacIntosh *et al*., 1996). Figure 3A also shows that the inhibitor TFP strongly decreased the AR formation compared with the H_2O-treated explants, suggesting that the signalling pathways under the control of endogenous pools of auxins and NO have been affected. Figure 3B displays representative photographs of AR formation in cucumber explants treated for 5 d as described above.

Table 1. *Effect of calmodulin antagonists on the CDPK activity induced by NO or IAA in cucumber explants*

Cucumber explants were incubated for different times with 10 μM SNP or 10 μM IAA in the presence (INH) or absence of a combination of both 100 μM of the calmodulin antagonists trifluoperazine dihydrochloride (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7). *In vitro* CDPK activity was measured in total soluble extracts from the hypocotyls of those explants. Data are from two independent experiments with two repetitions for each point. The *in vitro* CDPK activity was approximately 25 pmol ^{32}P min^{-1} mg^{-1} protein at any time in control H_2O-treated explants as well as at the moment of primary root removal (T_0).

Treatment	CDPK activity (pmol ^{32}P min^{-1} mg^{-1} protein) Days of treatment			
	1	2	3	4
SNP	42.7±3.5	62.4±8.7	96.9±8.7	89.9±5.2
SNP+INH	2.2±1.0	34.6±3.8	56.5±7.0	48.2±4.1
IAA	53.6±8.8	72.9±3.6	58.9±2.6	44.1±2.2
IAA+INH	4.5±2.1	2.3±0.9	28.5±1.5	2.4±1.0

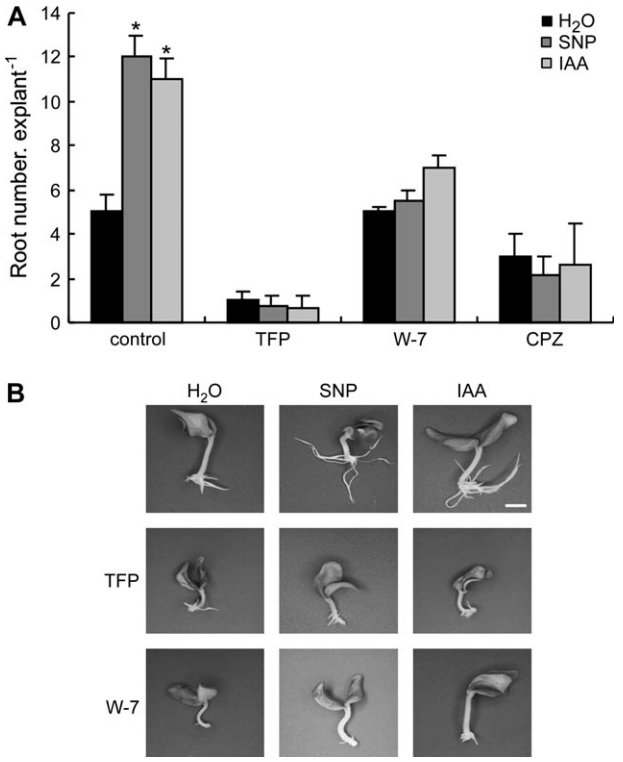

Fig. 3. Inhibition of CDPK activity blocks the NO- and IAA-induced adventitious root formation. (A) Cucumber explants were treated with 10 μM SNP or 10 μM IAA in the presence or absence of 100 μM of trifluoperazine dihydrochloride (TFP), 100 μM of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) or 100 μM of chlorpromazine hydrochloride (CPZ) for 5 d as indicated. Root number were quantified and expressed as mean ±SE (n=10 explants from at least three independent experiments). Bars with asterisk are significantly different (t test, P <0.05) from their control without SNP or IAA. (B) Photographs are representative for each treatment and were taken after 5 d of treatment. Bar indicates 5 mm.

The guanylate cyclase inhibitor LY83583 prevents the NO- and IAA-induced CDPK activity

According to a previous report, the IAA-induced AR formation is mediated by NO and requires the activity of the enzyme GC (Pagnussat *et al*., 2003). GC catalyses the synthesis of the second messenger cGMP and was shown to be positively regulated by NO in plants (Pfeiffer *et al*., 1994; Durner *et al*., 1998). To study the dependence on cGMP for the activation of CDPKs, the *in vitro* and in-gel CDPK activity was measured in soluble protein extracts of explants treated with the auxin IAA or with the NO-donor SNP in the presence or absence of 50 μM of the GC inhibitor 6-anilino-5, 8-quinilinedione (LY83583). It was previously shown that, at this concentration, LY83583 was able to block the effect of both IAA and NO in promoting AR formation in cucumber explants (Pagnussat *et al*., 2003). As shown in Fig. 4, the *in vitro* CDPK activity was strongly inhibited by the presence of LY83583. Furthermore, the in-gel protein kinase activity assays performed using extracts from SNP+LY83583- or IAA+LY83583-treated explants showed a total inhibition of the 50 kDa CDPK activity (data not shown). Finally, the LY83583-mediated inhibition of the CDPK activity was transiently reversed by the addition of the membrane-permeable cGMP analogue 8-Br-cGMP (data not shown). This evidence suggests that the CDPK activity is induced by IAA and NO in a cGMP-dependent pathway.

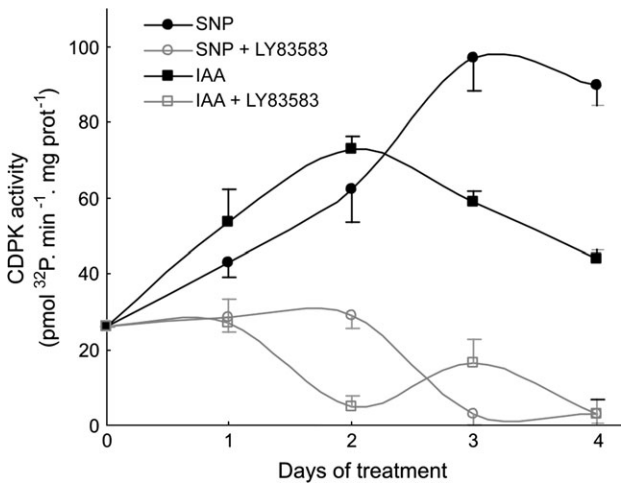

Fig. 4. The guanylate cyclase inhibitor LY83583 prevents the NO- and IAA-mediated induction of CDPK activity. Cucumber explants were incubated for different times with 10 μM SNP or 10 μM IAA in the presence or absence of 50 μM of the guanylate cyclase inhibitor 6-anilino-5,8-quinilinedione (LY83583). *In vitro* CDPK activity was measured in total soluble extracts from the hypocotyls of those explants. Values are expressed as pmol ^{32}P min^{-1} mg^{-1} protein. Data are from two independent experiments with two repetitions for each point.

Discussion

Activation of Ca^{2+}-mediated signalling pathways during adventitious root formation

cADPR and increases in $[Ca^{2+}]_{cyt}$ have been described as messengers in the cGMP-dependent signalling pathways induced by NO in both animals and plants (Lamattina *et al*., 2003; Neill *et al*., 2003; Stamler, 1994). In the present study, it was analysed whether the NO-induced and cGMP-mediated AR formation (Pagnussat *et al*., 2003) involves a cADPR/Ca^{2+}-dependent pathway. Data obtained from treating cucumber explants with compounds that are known to block cADPR/ryanodine-sensitive Ca^{2+} channels (methoxyverapamil hydrochloride and ruthenium red) or cADPR synthesis (nicotinamide) indicate that these inhibitors provoked a significant reduction in both IAA- and NO-induced AR formation. These results are consistent with a report that showed that the stimulation of *de novo* root formation by auxins in the monocotyledonous plant *Commelina communis* was suppressed by treatment with either (i) a GC inhibitor, (ii) a Ca^{2+} chelator, (iii) an inhibitor of Ca^{2+} release from intracellular stores, or (iv) an inhibitor of cADPR synthesis (Cousson, 2004). These findings suggest that cGMP could be implicated in Ca^{2+} mobilization by stimulating cADPR synthesis and subsequent Ca^{2+} release from cADPR/ryanodine-sensitive Ca^{2+} channels. Results indicate that this signalling pathway is also operative in a dicotyledonous plant during AR formation and that it relies on NO. Although the possibility cannot be excluded that some other Ca^{2+} channels might be affected due to the low specificity of the inhibitors methoxyverapamil hydrochloride and ruthenium red, the results presented in this work collectively argue that cADPR/ryanodine-sensitive Ca^{2+} channels are involved in the signalling pathways triggered by IAA and NO to induce AR formation. A similar process occurs in animals in which NO was shown to activate cADPR/ryanodine-sensitive Ca^{2+} channels indirectly via a cGMP/cADPR-dependent pathway (Willmott *et al*., 1996) or directly through *S*-nitrosylation (Xu *et al*., 1998). Besides the function of cGMP in mediating a cADPR/Ca^{2+}-dependent pathway, cGMP has also been shown to activate PM Ca^{2+} channels. These channels are called cyclic nucleotide-gated ion channels (CNGCs) and were identified throughout the plant kingdom. CNGCs are permeable to both monovalent and divalent cations (typically K^+, Na^+, and Ca^{2+}) and are directly activated by cGMP and/or cAMP (Lemtiri-Chlieh *et al*., 2004; Bridges *et al*., 2005).

Another pathway in which Ca^{2+} is released from intracellular stores is through IP_3-regulated channels (Alexandre *et al*., 1991; Allen *et al*., 1995). These data indicate that inhibitors of IP_3-regulated Ca^{2+} channels promoted a significant reduction in AR formation in both NO- and IAA-treated cucumber explants. In accordance, there is evidence that auxins could induce an increase in IP_3 concentration (Ettlinger and Lehle, 1988; Zbell and Walter-Back, 1988). It was also proposed that IP_3-regulated Ca^{2+} channels at the tonoplast might be involved in auxin-triggered increases in $[Ca^{2+}]_{cyt}$ (Shishova and Lindberg, 2004). In animals, it has been reported that NO could activate the enzyme PLC, which catalyses the formation of IP_3. Thus, PLC activity has been proposed to be part of the NO-dependent pathway that controls $[Ca^{2+}]_{cyt}$ via IP_3 (Clementi *et al*., 1995). The data presented in this study together with results obtained in this laboratory showing NO-dependent PLC activation in xylanase-elicited tomato (*Solanum lycopersicum*) cells (AM Laxalt *et al*., unpublished results), suggest that auxins and NO effects might also be accomplished through IP_3-regulated Ca^{2+} channels.

A recent report investigated whether cADPR/ryanodine- and IP_3-regulated Ca^{2+} channels exist in plants like the ones described in animals. On the basis of sequence analysis the authors found no homologous proteins in *Arabidopsis thaliana* and rice (*Oryza sativa*; Nagata *et al*., 2004). However, both types of channels have been implicated in different plant processes by biochemical, electrophysiological, and pharmacological studies. Similarly, neither nitric oxide synthase (NOS; an enzyme that catalyses NO formation) gene nor protein with sequence similarities to mammalian-type NOS has been found in the *A. thaliana* genome. However, pharmacological and biochemical studies demonstrated that NOS-like activities as well as inhibition of NO synthesis by inhibitors of mammalian NOS occurred in plants (Wendehenne *et al*., 2001). More recently, Guo *et al*. (2003) identified in *A. thaliana* a functional NOS gene that encodes a protein with sequence similarity to a protein that has been implicated in NO synthesis in the snail *Helix pomatia*.

Results indicate that the PM Ca^{2+} channel blocker lanthanum chloride significantly compromised the IAA- and NO-induced AR formation. Accordingly, in a recent study it was reported that NO can regulate changes in $[Ca^{2+}]_{cyt}$ through the control of Ca^{2+} influx across the PM in tobacco cells (Lamotte *et al*., 2004). In the same direction, auxin might activate Ca^{2+} transport from the extracellular space through PM Ca^{2+} channels as shown in wheat (*Triticum aestivum*) leaf protoplasts (Shishova and Lindberg, 2004). The requirement of extracellular Ca^{2+} to promote AR formation in cucumber explants was corroborated via the use of the membrane-impermeable Ca^{2+} chelator EGTA. Notwithstanding the limitations of the pharmacological approach used in this study, these data collectively indicate that both intracellular and extracellular Ca^{2+} pools are required for the action of IAA and NO in triggering AR formation. Thus, in addition to the function of Ca^{2+} as a mineral nutrient modulating the root growth (Druart, 1997; Bellamine *et al*., 1998), these results provide evidence to support the involvement of Ca^{2+} as a second messenger linking both auxins and NO to the activation of processes leading to AR formation.

CDPK activity is induced by IAA and NO during adventitious root formation

CDPKs are involved in signalling pathways that utilize changes in $[Ca^{2+}]_{cyt}$ to couple cellular responses to extracellular stimuli (Roberts and Harmon, 1992). An increase in a 50 kDa CDPK activity is reported here after 1 d of exposure of the cucumber explants to either the NO-donor SNP or the auxin IAA. The specific NO-scavenger cPTIO delayed the CDPK activity in SNP-treated explants and diminished it in IAA-treated ones. These results suggest that the 50 kDa CDPK detected by in-gel assays in both NO and IAA treatments is the same and requires the presence of NO for its maximal and sustained activity. The induction of CDPK gene expression in response to auxins was previously reported in alfalfa (*Medicago sativa*; Davletova *et al*., 2001). In the present study auxins and NO are put together in the Ca^{2+}-mediated signalling pathway that regulates CDPK activity leading to AR formation. The rate of NO release from the NO-donor SNP displays a peak around the second day in aqueous solutions (MV Beligni and L Lamattina, unpublished results). This fact might explain the partial inactivation of the 50 kDa CDPK in SNP+cPTIO treatment at the second and third days of treatment. With regard to the auxin action, a transient increase in the endogenous NO level was reported to occur during the first day of IAA-treated cucumber explants (Pagnussat *et al*., 2002). Thus, the amount of cPTIO could be insufficient to scavenge the NO entirely and therefore to inhibit the CDPK activity completely during the first and second days of IAA+cPTIO treatment. The different dynamics of CDPK activity measured by *in vitro* and in-gel assays could be explained by the nature of the experiments. While in the *in vitro* assays the total CDPK activity is measured, in the in-gel assays only the CDPKs that are able to renature under the experimental conditions are detected. In addition, syntide 2 is used as substrate for *in vitro* assays and histone IIIS for in-gel analysis.

Overall, Fig. 5 summarizes the available data that suggest a scenario in which basipetal transport of auxins induces an NO burst in the basal region of the cucumber hypocotyl, where the AR primordia develop (Pagnussat *et al*., 2002). Then, NO triggers a bifurcated signalling pathway that includes: (i) increases in the levels of cGMP, cADPR, and IP_3 that results in elevations in both $[Ca^{2+}]_{cyt}$ and CDPK activity; and (ii) the induction of a cGMP-independent MAPK cascade (Fig. 5; Pagnussat *et al*., 2003, 2004). It would be noteworthy that the compounds TFP, W-7, and CPZ are not specific for inhibiting CDPKs. They also affect the activity of other Ca^{2+}-binding proteins such as CaM and calcineurin B-like proteins (Anil and Rao, 2000). However, the model presented in Fig. 5 is in accordance with these results showing an inhibition of the CDPK activity by those compounds in both *in vitro* and in-gel protein kinase assays. Nevertheless, this does not preclude the possibility that inhibition of other proteins by TFP, W-7, and CPZ could also be affecting AR formation. The activation of all the pathways triggered by NO seems to be required for AR formation since the stimulatory effect of auxins and NO is abolished when one of the pathways is compromised. It remains to be determined whether co-ordination and/or synchronization between MAPK- and Ca^{2+}/CDPK-dependent signalling pathways occur during AR formation.

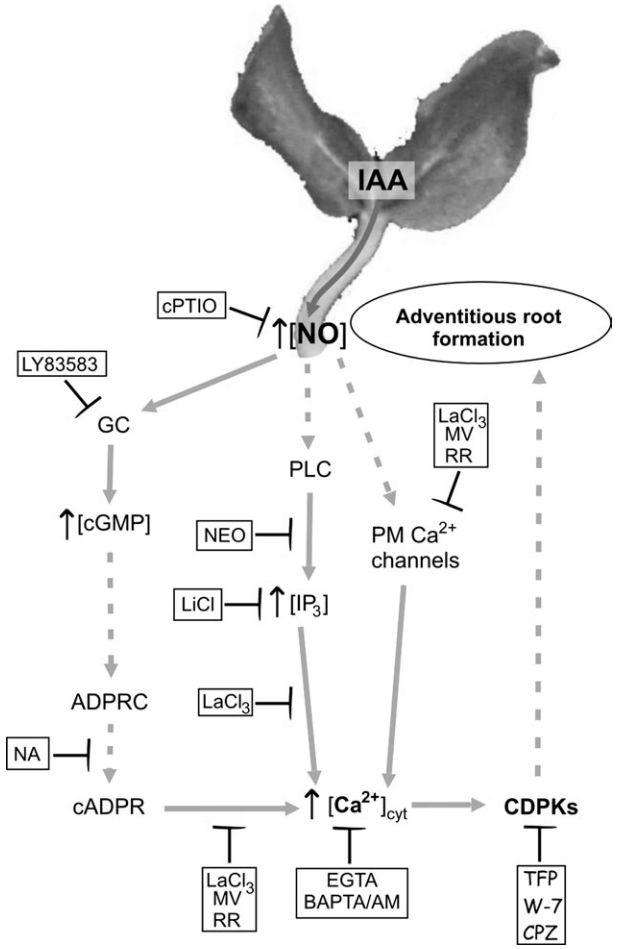

Fig. 5. Schematic representation of the signalling pathways induced by IAA and NO during adventitious root formation in cucumber. NO accumulates in the basal region of the hypocotyls in response to basipetal transport of IAA (Pagnussat *et al*., 2002). NO induces an increase in $[Ca^{2+}]_{cyt}$ through the regulation of both the release of Ca^{2+} from intracellular stores (via cADPR/ryanodine- and IP_3-regulated Ca^{2+} channels) and the influx of Ca^{2+} from the extracellular space (via PM Ca^{2+} channels). As a consequence, certain CDPKs become activated. These protein kinases could probably contribute to the downstream responses that result in AR formation. cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; GC, guanylate cyclase; ADPRC, ADP-ribosyl cyclase; cADPR, cyclic adenosine diphosphoribose; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; PM, plasma membrane; CDPKs, Ca^{2+}-dependent protein kinases; ⊥, inhibition. Dashed lines denote indirect or still undescribed pathways, or uncharacterised proteins. All the inhibitors assayed in this study are boxed.

The isolation of five cDNAs encoding cucumber CDPKs provided evidence for the existence of multiple isoforms and raises the question of the physiological function of each one (Ullanat and Jayabaskaran, 2002; Kumar *et al*., 2004). In this study, the activity of the 50 kDa cucumber CDPK was detected at earlier stages of AR formation, suggesting that this protein kinase could be associated with cell de-differentiation, division and/or differentiation. These results fit with those previously reported by Kobayashi and Fukuda (1994) and by Komatsu *et al*. (1996), in which Ca^{2+}-binding proteins and protein kinases were found to be involved in plant differentiation processes. Future studies including cell type-, tissue- and organ-specific expression profiles, identification of specific substrates, and subcellular localization will be necessary to elucidate the biological function of each cucumber CDPK isoform.

Most eukaryotic signalling pathways involve spatially and temporally specific elevations in $[Ca^{2+}]_{cyt}$, either via release from intracellular stores and/or via influx from the extracellular space, being both processes closely related. The other major mechanism in cell signalling is the reversible protein phosphorylation, including protein kinases and phosphatases (Neill *et al*., 2003). It seems that these two signalling events are activated during the NO-induced AR formation and may represent key components of the NO responses in plants.

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